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Fertility preservation: potential ovarian protective effect of GnRH analogues during chemotherapy

Márcio André Gonçalves Madureira

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MASTER THESIS

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Abstract

Recent advances in cancer treatment field allowed significant increase in the survival rate of patients. However, the patients have commonly faced long-term adverse effects that severly affected the quality of life, specially concerning their fertility. It is established that radio- and chemotherapy treatments can cause a reduction of the ovarian reserve, resulting in a 40-60% rate of premature ovarian failure (POF) in women exposed to these treatments. In order to decrease POF risks, several fertility preservation options were developed: reduction of the exposure to gonadotoxic agents, gametes or embryos cryopreservation, oocyte donation, ovarian tissue cryopreservation and transplant, or a pharmacological protection of the ovaries during chemotherapy. While the last one appears as a less invasive and promising procedure, the studies and clinical trials continue to show inconsistent results, raising an almost 30-year discussion and controversy. The gonadotropin-releasing hormone (GnRH) is a hypothalamic hormone responsible for the releasing of gonadotropins. Due to its pulsatile fashion to induce FSH secretion, it was proposed that a continuous saturation of the receptor by synthetic GnRH analogues (GnRHa) could decrease the gonadotropins release and, therefore, could maintain the ovarian follicular pool at immature stages. Once it was suggested that initial stage follicles were less affected by alkylating agents, the women capacity to conceive could then be preserved. By a multiple approach study design, including histological, immunohistochemical, *in vitro* and *in vivo* assays in a mouse model, our group intended to better understand the potential preventive effect of GnRHa on the ovaries exposed to chemotherapy and to evaluate the efficiency of GnRH agonists (AGOs) and antagonists (ANTs) in this indication. Our results suggest, so far, that AGOs (triptorelin) and ANTs (cetrorelix) are not efficient to prevent the follicular depletion induced by a cyclophosphamide (Cy) treatment. Nevertheless, the fertility follow-up, until now, seems to show that the birth rate is not affected by neither Cy nor combined Cy-GnRHa treatment, suggesting that the mouse experimental model is not yet optimal.

Keywords: fertility preservation, chemotherapy, GnRH agonists, GnRH antagonists

Resumo

Nos últimos anos, foram feitos enormes avanços no que diz respeito aos tratamentos oncológicos. Estes conseguiram aumentar significativamente as taxas de sobrevivência e o aumento da esperança média de vida dos doentes oncológicos. No entanto, este incremento nem sempre foi acompanhado por uma melhoria na qualidade de vida, nomeadamente na preservação da fertilidade das mulheres sujeitas a tratamentos radio- e quimioterapeuticos. Em média, após este tipo de tratamentos, 40-60% das mulheres são diagnosticadas com falência ovárica precoce (FOP), caracterizada essencialmente por uma diminuição acentuada no número de folículos pertencentes à reserva ovárica de mulheres ainda em idade-reprodutora, derivando consequentemente em amenorreia e, portanto, perda da capacidade de engravidar. Em suma, a FOP é essencialmente definida como uma menopausa prematura em mulheres com idade inferior a 40 anos. A alteração hormonal daí decorrente é comummente associada a um aumento no risco de aparecimento de outras perturbações clínicas, como a osteoporose, doenças cardivasculares e depressão. Desta forma, os serviços oncológicos hospitalares, em parceria com os serviços de ginecologia e obstetrícia, pretenderam desenvolver métodos de prevenção à FOP. A investigação decorrente permitiu a criação de diversas opções à preservação da fertilidade, tais como: a redução da exposição aos agentes gonadotóxicos, a criopreservação de oócitos, embriões ou mesmo de tecido ovárico para posterior transplantação, doação de gâmetas, ou ainda a protecção farmacológica dos ovários durante o tratamento quimioterapeutico. Entre estes, a protecção farmacológica surge como uma excelente opção para a recuperação expontânea da actividade ovárica, uma vez que se trata de um procedimento menos invasivo. Contudo, as conclusões resultantes de diversos estudos e ensaios clínicos geraram uma substancial controvérsia, na medida em que não surgiram ainda dados concretos e consistentes da efectividade dos fármacos na protecção ovárica. Apontados como o produto com maior potencial na prevenção da FOP, a acção de substâncias análogas (agonistas e antagonistas) da *gonadotropin-releasing hormone* (GnRH) tem sido amplamente estudada, tanto em modelos animais como em ensaios clínicos em humanos. A GnRH é uma hormona sintetizada no hipotálamo, cuja função é o controlo da secreção das gonadotropinas FSH e LH na hipófise anterior. A produção de FSH e LH está dependente de um estímulo de carácter pulsátil da GnRH. Visto isto, a exposição permanente dos receptores hipofisários aos análogos da GnRH (GnRHa) origina uma inibição na secreção das gonadotropinas, que desta forma não acederão ao ovário,

impedindo o normal desenvolvimento folicular. Como anteriormente referido existem duas classes de GnRHa: agonistas (AGOs) e antagonistas (ANTs). Os AGOs têm relativamente poucas alterações em comparação com a hormona natural. São igualmente decapéptidos, cujas modificações ocorrem essencialmente ao nível do aminoácido na 6ª posição (glicina), que aumenta o tempo de meia-vida da hormona, protegendo-a da degradação por peptidases; na 10ª posição (glicina carboxiloterminal), que melhora a afinidade do GnRHa ao receptor (GNRHR). Relativamente aos ANTs, estes podem conter múltiplas alterações, ocorrendo sobretudo nas três primeiras posições – região de ligação ao GnRHR. Os GnRHa foram desenvolvidos com vista à alteração da produção de gonadotropinas. Os AGOs induzem primeiramente uma forte secreção, no entanto a contínua administração provoca uma saturação do complexo AGO-GnRHR, originando uma queda acentuada na concentração de FSH e LH circulantes (produzidas e excretadas apenas na existência de uma actividade pulsátil da GnRH). Por outro lado, os ANT actuam por via duma competição com a hormona natural pelo GnRHR, bloqueando estes e suprimindo assim a normal actividade da GnRH na hipófise. Alguns autores propõem que esta actividade inibitória, de ambos os GnRHa, é capaz de proteger a reserva ovárica dos efeitos da quimioterapia, uma vez que esta incide sobretudo nos folículos que se encontram em desenvolvimento. Ou seja, a criação de condições hipopituitárias, poderá impedir a secreção de FSH – hormona essencial ao crescimento e ao recrutamento folicular –, e assim impossibilitar a acção de agentes quimioterapeuticos, cuja intervenção incide sobretudo nas células com actividade proliferativa. Concretamente, a nível das células da granulosa – células do folículo ovárico de maior actividade mitótica.

As primeiras demonstrações de um possível efeito protector dos GnRHa num ovário exposto a quimioterapia ocorreram nos anos 80, em experiências que utilizavam o rato como modelo. Estas apresentaram resultados animadores, uma vez que o AGO utilizado conseguia diminuir a depleção de folículos ováricos de ratos tratados simultaneamente com ciclofosfamida (agente quimioterapeutico). Posteriormente, um estudo com macacos rhesus demonstrou que a ciclofosfamida destruía 65% dos folículos primordiais, enquanto que um co-tratamento com AGO reduzia esta população folicular em apenas 29%. No entanto, outros estudos demonstraram diferenças não significativas entre as várias condições analisadas, levantando então dúvidas relativamente ao sucesso dos AGOs na preservação da fertilidade. Comparativamente, o uso de ANTs em modelos animais expostos a tratamentos quimioterapeuticos, demonstrou alguns casos promissores, mas não na totalidade. Um estudo recente, apresentou uma menor depleção da reserva folicular

em ratinhos co-tratados com ciclofosfamida e cetrorelix (ANT), quando comparada com os ratinhos expostos apenas à ciclofosfamida. Contudo, outro estudo afirmou que, embora a diferença nas contagens de folículos efectivamente ocorresse, a diminuição não se repercutia posteriormente numa diferença significativa no número de crias por ninhada, nas várias condições analisadas. No que respeita à investigação em humanos, diversos estudos foram já realizados, porém com a controvérsia da acção dos GnRHa na protecção do ovário a manter-se. As conclusões de inúmeros estudos foram postas em causa, devido à metodologia utilizada e ao carácter incompleto de alguns ou pelo reduzido número e em enviesamento da amostra de outros. No entanto, ensaios clínicos mais recentes, cuja pretensão se centrou na avaliação mais rigorosa do efeito dos GnRHa, continuam a fornecer resultados preliminares e conclusões inconsistentes e díspares uns dos outros. Mantendo-se assim a discussão sobre o verdadeiro efeito dos GnRHa nos ovários de mulheres submetidas a quimioterapia, e desconhecendo-se igualmente os mecanismos fisiológicos subjacentes à acção destes.

Visto isto, o presente estudo pretendeu melhor elucidar o potencial efeito protector dos GnRHa nos ovários durante a quimioterapia, assim como comparar a eficácia de AGOs e ANTs no desempenho desta função preventiva. Recorrendo ao ratinho como modelo, este estudo contem diferentes abordagens, de forma a tentar responder com complementaridade a diversas questões, avaliando assim de forma mais generalizada o papel dos GnRHa: a reserva ovárica foi avaliada através de contagem folicular por fase de desenvolvimento, recorrendo a técnicas histológicas (coloração hematoxilina e eosina); as taxas de proliferação celular e apoptose foram avaliadas por imunohistoquímica (ki-67 e caspase-3, respectivamente); o desenvolvimento folicular foi analisado através de culturas *in vitro* (12 dias em meio de crescimento + 1 dia em meio de maturação, e consequente desnudagem e avaliação do estado de maturação do oócito); a fertilidade foi estudada a partir do acompanhamento das ninhadas produzidas; a competência do oócitos foi verificada pela técnica de fertilização *in vitro* (embora os resultados não sejam apresentados neste estudo). Seis condições de tratamento foram definidas: controlo – aos ratinhos fêmea foi diariamente administrada uma injecção subcutânea (sc) de uma solução salina (NaCl) e uma única injecção intraperitoneal (ip) foi aplicada, igualmente, com NaCl; controlo-quimioterapia – sc diária NaCl e uma única ip de ciclofosfamida (Cy); controlo-ANT – sc diária cetrorelix e uma única ip NaCl; ANT-quimioterapia – sc diária cetrorelix e uma única ip Cy; controlo-AGO – sc diária triptorelin e uma única ip NaCl; AGO-quimioterapia – sc diária triptorelin e uma única ip Cy. Os ratinhos fêmea receberam tratamento base durante 21 dias e a ip foi administrada no dia 13. No dia

do sacrifício foi recolhido sangue, com recurso a uma punção intracardíaca; a cada dois dias das culturas foliculares *in vitro* o meio de cultura era colhido, renovado e armazenado. Todas as colheitas armazenadas serviram posteriormente para que análises hormonais fossem efectuadas. A comparação entre o efeito produzido pelo AGO e ANT na reserva folicular, demonstrou que o grupo controlo foi aquele cujo número de folículos em estados iniciais – primordiais e primários – era mais elevado (441 ± 153). Embora as diferenças notadas não tenham atingido valores estatisticamente significativos (p=0,358), o grupos controlo-ANT e controlo-AGO apresentaram um decréscimo, respectivamente, de 17% e 23%, em comparação com o controlo. Relativamente aos grupos tratados com Cy, todos demonstraram novamente valores proporcionais inferiores (comparativamente ao controlo): Cy – menos 32%; ANT+Cy – menos 39%; AGO+Cy – menos 44% (o único a atingir valores significativos, p=0,022). Os folículos em crescimento – secundários, *early* antrais e antrais – apresentaram contagens sem diferenças significativas entre todas as condições. A proporção relativa dos vários estadios de desenvolvimento folicular demonstraram valores muito próximos, entre todas as condições. A percentagem de folículos em estados iniciais variou entre 66-78% e a de folículos em crescimento 22-34%. Testes imunohistoquímicos preliminares à proteína ki-67 parecem marcar preferencialmente as células da granulosa de folículos em crescimento. No que concerne as culturas foliculares *in vitro*, a taxa de sobrevivência foi semelhante em todas as condições, assim como na taxa de maturação ovocitária. Todas as condições apresentaram uma taxa de oócitos em meiose II entre 51-67%. A produção de progesterona (24h) também não apresentou diferenças significativas entre condições, tendo o grupo controlo revelado uma produção de 2,89 ng/mL. O acompanhamento dos nascimentos ocorridos não demonstrou, até ao momento, nenhuma diferença entre os vários grupos analisados.

Os resultados até agora obtidos parecem sugerir uma ausência de efeito protector na reserva folicular, aquando o uso de GnRHa em ovários expostos a ciclofosfamida. No entanto, o projecto encontra-se ainda a decorrer, pelo que mais e melhores informações deverão elucidar de forma mais clara o verdadeiro papel dos GnRHa no ovário submetido a tratamento quimioterapeutico.

Palavras-chave: preservação da fertilidade, quimioterapia, agonistas da GnRH, antagonistas da GnRH

Contents

Introduction

Over the last years, the progresses in the cancer treatment field allow to remarkably improve survival rates among patients. A recent report from the Office for National Statistics of the United Kingdom (UK) says that, presently, near 10% of women face or will face breast cancer – the most common malignancy in adult women; however, the 5-year survival rate for women treated for breast cancer in the UK is now above 80% (ONS, 2010). The development in radio- and chemotherapy treatments created, nonetheless, a clinical concern related to the long-term adverse effects of cancer treatments. Chemotherapy-treated women are frequently diagnosed with early menopause, or with an increase in infertility rate even in those who recover their ovarian function after chemotherapy. These adverse events dramatically affect their quality of life (Letourneau *et al.,* 2012). The premature ovarian failure (POF) has also been related to an increase risk of osteoporosis (Bruning *et al.,* 1990), cardiovascular diseases (Jeanes *et al.,* 2007) and psychosocial problems, such as depression (Carter *et al.,* 2005). This early menopause is specifically characterised by a premature depletion of functional ovarian follicles leading to the arrest of the menstrual cycle – amenorrhea – in women of reproductive age (Goswami *et al*., 2005). It is usually established that 40-60% of women diagnosed with invasive cancer will face POF (Meirow, 2000). Therefore, fertility preservation in reproductive-aged women became a major concern in oncologic units.

Several options for the preservation of women fertility have been developed, such as: reduction of the gonadotoxic treatments, cryopreservation of gametes or embryos, oocyte donation, ovarian tissue cryopreservation and transplant, or even a pharmacological protection of the ovaries during chemotherapy (Figure 1). For medical or personal (or also legal) reasons, the established fertility preservation methods are not always available or indicated to all patients.

Regarding these issues, pharmacological protection could represent an interesting option to increase the chances of spontaneous ovarian function recovery after chemotherapy, avoiding more invasive procedures. The administration of the *Gonadotropin-releasing hormone* (GnRH) agonist has been proposed as potential “ferto-protective” therapy during chemotherapy.

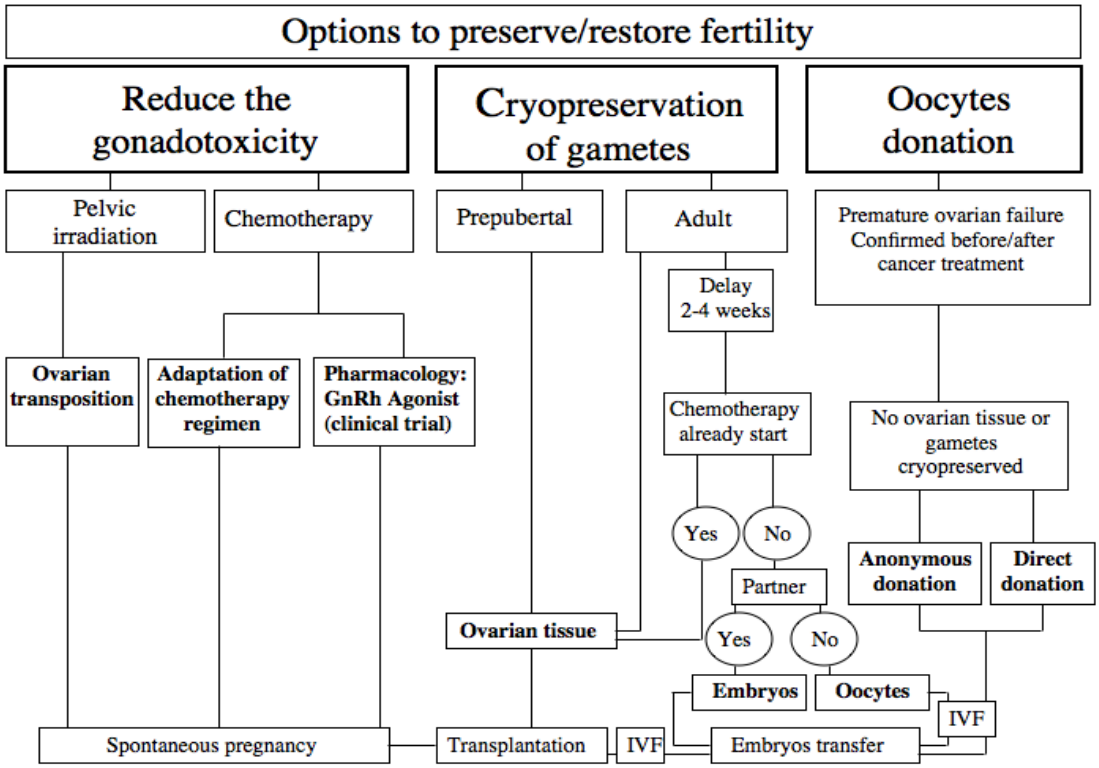


Figure 1: Different options available for cancer patients on order to preserve their fertility (Demeestere *et al.,* 2007).

The identity and structure of the GnRH were first described in the work of Schally and his colleagues (Schally *et al.,* 1971). Among the key events, after this discovery, authors described the pulsatile fashion of GnRH release in the circulation (Knobil, 1974) and demonstrated that these pulses are crucial to maintain the synthesis and secretion of gonadotropins – follicle-stimulating hormone (FSH) and luteinizing hormone (LH) – and so the reproductive function (Knobil, 1980).

The core of GnRH-secreting neurons is located in the medial basal hypothalamus (Figure 2). Once released, the GnRH penetrates directly on the hypophyseal portal system vessels and reaches the anterior pituitary. Then, the GnRH binds to a receptor (GnRHR) expressed in gonadotropic hypophyseal cells that subsequently secrete FSH and LH (Naor, 2009). The gonadotropin secretion stimulus is dependent on this GnRH pulsatile release, once that the continuous exposure of the receptors to GnRH downregulates the LH and FSH secretion (Bédécarrats *et al.,* 2003).

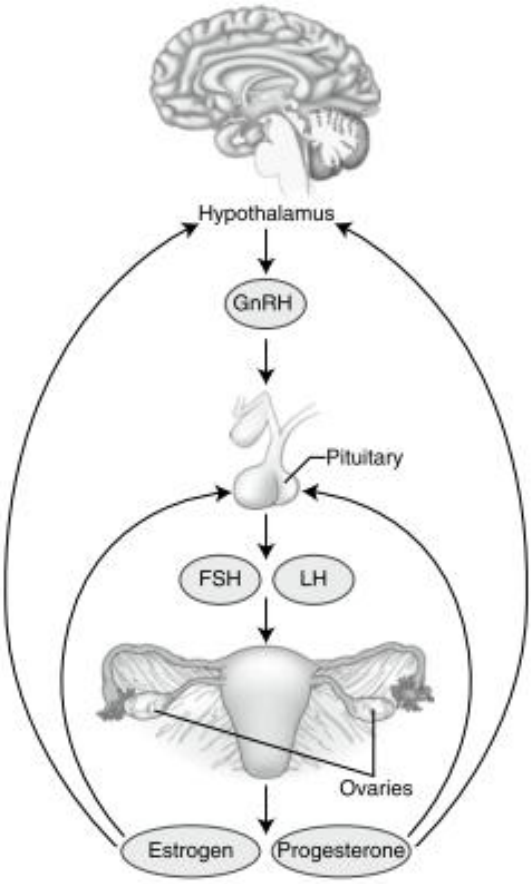


Figure 2: Hypothalamic-hypophyseal-gonadal axis (Cakmak & Seli 2012).

Two classes of GnRH analogues where synthetized as a therapeutic option for different diseases: GnRH agonists (AGOs) and GnRH antagonists (ANTs). The human GnRH is a decapeptide with the following amino acid sequence: Glutamic acid – Histidine – Tryptophan – Serine – Tyrosine – Glycine – Leucine – Arginine – Proline – Glycine (Figure 3).

Regarding GnRH agonists, they have relatively few modifications compared to the native GnRH. Modifications concern the glycine at position 6 and the carboxyterminal glycine at position 10. The modification at position 6 increases the half-life of the molecule by protecting the hormone from degradation, since this position is the target of peptidases. The modification at position 10 increases the receptor affinity by 100-200 times (Shapiro, 2003).

On the other hand, GnRH antagonists have multiple substitutions. Among them, the majority are on the amino acids at the positions 1, 2 and 3, the receptor-binding region (Shapiro, 2003).

Figure 3: GnRH amino acid sequence.

These synthetic drugs (the GnRHa) were designed to modify the release of gonadotropins. As previously mentioned, GnRH agonists have similar structure compared to native GnRH and a higher affinity to the GnRHRs. Firstly, they induce gonadotropins release (flare-up effect), but after a continuous administration they result in a dramatic drop of the circulating concentrations of FSH and LH through a desensitization mechanism. GnRH agonists have a greater affinity for the GnRHR than native GnRH, a greater resistance to enzymatic breakdown and a prolonged half-life compared to native GnRH (in humans, native GnRH has a half-life of 2–4 min compared to 3h for GnRH agonist, leuprolide) (Chillik *et al.,* 2001). The prolonged saturation of the AGO–receptor complex leads to a profound inhibition of the gonadotropins secretion (Ortmann *et al.,* 2002). Although they have the same function, GnRH antagonists act through different mechanisms causing immediate gonadotropin release suppression by competitively blocking GnRHRs in the pituitary (Horvath *et al.,* 2002).

Some authors suggested that GnRHa may protect the ovary during chemotherapy thanks to their inhibitory effect on gonadotropins secretion and therefore on the ovarian function. The presence of FSH is indeed essential for follicular growth (Gougeon, 1996). During hypopituitary conditions, the follicular growth is partially abolished. Low FSH levels induced by GnRHa could also inhibit the process of recruitment from the pool of small follicles to the pool of larger follicles. This is suggested by a study analysing the $[Thymidine]^3$ incorporation into ovarian DNA, which demonstrated that GnRHa could suppress granulosa cells mitotic activity (Ataya *et al.,* 1988). Dividing cells are known to be more sensitive to the cytotoxic effects of chemotherapeutic agents than the cells at resting stage. Furthermore, high levels of gonadotropins are associated with an increase of the normal follicular atresia process. This is confirmed by the following observation: in postnatal normal rodents, plasma FSH is elevated up to 18 days of life. During this period, the remaining follicular stock decreases by half (Gougeon, 1996). Inversely, low levels of gonadotropins encountered in hypophysectomy decrease the normal process of progressive loss of oocytes. Elevated gonadotropin serum levels frequently observed during chemotherapy

could produce an increase of the follicular atresia process. By inhibiting the gonadotropin secretion, GnRHa could reduce the rate of atresia and protect the ovary during chemotherapy.

The effectiveness of the GnRH agonist treatment was first reported in rats and monkeys in the 1980s. Studies on rats treated with Cyclophosphamide (Cy) combined with AGO showed a decrease in the chemotherapy-induced follicular depletion by maintaining the follicular pool at the resting stage (Ataya *et al.,* 1988; Ataya *et al.,* 1989). Experiments on monkeys demonstrated that 65% of the primordial follicular pool is destroyed after Cy treatment compared to 29% after Cy+AGO treatment. The primordial follicles declining rate per day is significantly reduced in the combined treatment group compared to the Cy treatment group (0.06% vs. 0.12%) (Ataya *et al.,* 1995). Tan and his fellows obtained a greater number of primordial and primary follicles when a high-dose of agonist was applied simultaneously with a busulfan treatment (Tan *et al.,* 2010). In contrast, Montz *et al.,* reported that Lupron (an AGO) was able to preserve fertility in rats exposed to the gonadotoxic effect of Cy, but failed to protect fecundity (Montz *et al.,* 1991). Concerning the co-treatment of GnRH antagonists along with chemotherapy, Meirow's group showed in 2004, a promising study where the co-administration of cetrorelix contributed to a minor ovarian damage and greater primordial follicles count when compared with the Cy-only group, on a mice model (Meirow *et al.,* 2004). In the study of Lemos *et al.*, they found a histological significant difference in the follicular count of the group of rats treated only with ANT (compared with the control), however the number of pups that were born were not statistically different (Lemos *et al.,* 2010). The efficiency of these treatments was however seriously debated since many years. Other authors support that the treatment cannot efficiently inhibit the initial activation and growth of the primordial follicles as this phase is gonodotrophins independent (Oktay *et al.,* 2007). Furthermore, others study did not show any benefit of the administration of GnRha concomitantly to the chemotherapy on the fertility (Montz *et al.,* 1991) and even showed a detrimental effect (Maltaris *et al.,* 2007).

In humans, the efficiency of GnRHa in preventing premature ovarian failure remains also controversial. Some non-randomized studies suggested a reduction of premature ovarian failure rate when AGO was administered concomitantly to the chemotherapy (Blumenfeld *et al.,* 2008; Castelo-Branco *et al.,* 2007; Dann *et al.,* 2005; Huser *et al.,* 2008; Pereyra Pacheco *et al.,* 2001). However, the methodology of these studies was criticized, thus calling the results into question (Beck-Fruchter *et al.,* 2008).

After the observation, by some, of a beneficial effect of GnRH agonists on future ovarian function, new, larger and prospective randomized studies have been initiated. In 1987, Waxman and colleagues demonstrated that buserelin (AGO) was ineffective in conserving fertility in a group of Hodgkin's lymphoma patients (Waxman *et al.,* 1987).The *Gruppo Italiano Mammella* indicated a reduction of 17% in the occurrence of POF in the cohort group treated simultaneously with chemotherapy and triptorelin (Del Mastro *et al.,* 2011). On the other side, also resorting to breast cancer patients (like the previous mentioned study), Munster *et al.,* reported comparable amenorrhea rates in the triptorelin/chemotherapy-treated and control groups (Munster *et al.,* 2012). In 2010, the *German Hodgkin Study Group* presented an ovarian follicle preservation rate of 0% in all studied groups, in a clinical trial where oral contraceptives and goserelin were independently tested for the ovary protection during an escalated combination chemotherapy regimen in advanced-stage Hodgkin lymphoma patients (Behringer *et al.,* 2010). Likewise, Demeestere *et al.,* are currently at the end of the first year of follow-up of a multicentre, randomised, prospective trial including Hodgkin's and non-Hodgkin's lymphoma patients, and the results show approximately 20% of POF either in the triptorelin co-treated or in the control arm (Demeestere *et al.,* 2012)

Nevertheless, despite some of these clinical trials suggest an effect of GnRH agonist to protect the ovarian reserve of patients who spontaneously recover their ovarian function, the physiological mechanisms of ovarian protection is still poorly described and controversial.

Therefore, our project intended to contribute for a better understanding of the protective effect GnRHa on the ovary during chemotherapy and to compare the efficiency of GnRH agonist and GnRH antagonist using mice model. The study had several different approaches, in order to evaluate: the ovarian reserve (histological follicular count), the follicular proliferation and the apoptotic rates (immunohistochemistry), the follicular developmental potential (*in vitro* follicular culture system), the fertility (follow-up of the litter size) and oocyte competence (IVF).

Material and Methods

Animals

All experiments were performed using F1 females hybrid mice (C57blxCBAca, Harlan, The Netherlands) aged 6-8 weeks at the beginning of the treatments. The animals had free access to food and water, and they were kept under the specific conditions approved by the institution's ethics committee.

Drug treatments

The experiments were designed to compare 2 different GnRH analogues (GnRHa): cetrorelix acetate - GnRH antagonist (ANT) (Cetrotide®, Merck Serono, Switzerland); triptorelin acetate - GnRH agonist (AGO) (Gonapeptyl®, Ferring Pharmaceuticals, Switzerland). For the chemotherapy, an alkylating agent was used: Cyclophosphamide monohydrate (Cy) (Sigma-Aldrich, USA)

Each experiment was conducted using two female mice per condition, who received the same treatment: a daily 100 µl subcutaneous injection of the GnRHa (0,5 mg/kg) or vehicle only (NaCl, saline solution) during twenty-one days and a single 100 µl intraperitoneal injection of Cy (75 mg/kg) or vehicle on day 14. This dose of chemotherapy is supposed to destroy half of the ovarian reserve (Meirow, 1999). The conditions included the following groups:

- Control (vehicle only)
- Chemotherapy-control (daily vehicle and once Cy)
- Antagonist-control (daily ANT and once vehicle)
- Antagonist-chemotherapy (daily ANT and once Cy)
- Agonist-control (daily AGO and once vehicle)
- Agonist-chemotherapy (daily AGO and once Cy)

The injected doses were chosen based on previous studies that showed significant ovarian dose-response effects (D Meirow 1999; Dror Meirow *et al.,*. 2004).

Peripheral blood intra-cardiac puncture

On the 21st day of the treatments, all the females (with the exception of those from the follow-up assay) were anesthetised with an intra-peritoneal injection of saline solution containing 10% xylazine 2% (Rompun®, Bayer, Germany) and 20% ketamine

HCl (Ketamine 1000®, Ceva, Belgium), and an intra-cardiac puncture (IC) was made with a 26-gauge needle to collect peripheral blood. A mean of 600 µl of blood per mouse was collected to Eppendorf® tubes, and then centrifuged at 13 x 10^5 rpm for 10 min, so that the blood serum could be saved and used in further hormonal assay. The mice were sacrificed immediately after the IC by cervical dislocation.

Follicular count

Subsequently to the IC and cervical dislocation, the mice were ovariectomised. For each mouse, one ovary was fixed in 4% paraformaldehyde overnight, embedded in paraffin and serially sectioned in the microtome into 5 µm slices. Sections were stained resorting to Haematoxylin–Eosin coloration and the number of follicles was counted and properly classified in every 5th section. Follicles were classified according to the granulosa cells layers and counted only when the oocyte nucleus was visible in the section. The follicular development and classification was divided into five stages (Figure 1): primordial – with a single flat granulosa cells layer; primary – with single layer of cuboidal granulosa cells; secondary – more than 1,5 layers of granulosa cells; early antral – multiple granulosa cells layers and presence of cavity(ies), synonym of the formation of an antrum; antral – antrum fully formed, with the oocyte located already on the periphery of the follicle surrounded by the cumulus cells (cumulus oophorus). The counting was made unaware of which condition was being analysed. The final results do not present any kind of extrapolation or correction factor, which means that the values showed on this study can't be seen as a representation of the whole ovary.

Figure 4: Classification of the different follicles developmental stages in adult mice ovaries

Preantral follicles culture

The other ovary was dissected using a 26-gauge needle in order to isolate

preantral follicles. Only intact preantral follicles, with diameter between 100-130 μm and characterized by at least two complete granulosa cell layer and a visible centrally located oocyte, were selected for culture. The culture medium is composed of MEM Glutamax supplemented with 5% FBS, 1% ITS (Insulin 5 μg/ml, Transferrin 5 μg/ml, Selenium 5 ng/ml), 1% r-LH (80 mIU/ml), 0,1% r-FSH (100 mIU/ml). Each selected follicle was rinsed, individually transferred in a microdrop of 10 μl of culture medium under mineral oil and cultured at 37°C in a humidified atmosphere of 5 % $C0_2$ in air. After two days of culture, 10 μl of fresh medium were added in each drop. Every 2 days until day 12 of culture, 10 μl of medium of each drop was collected and replaced by fresh medium. The collected culture media from the drops containing surviving follicles was pooled and stored at – 20°C until further hormonal assays. After 12 days of culture, oocyte maturation was induced by refreshing the medium with maturation medium. The maturation medium consisted of culture medium supplemented with 1,5 IU/ml r-hCG and 5 ng/ml EGF. Sixteen to eighteen hours post r-hCG/EGF, oocyte-cumulus-complexes (OCC) were collected and mechanically denuded to evaluate the oocyte nuclear maturation stage. Three stages were defined according to the maturation and temporal development of the oocyte (Figure 2): germinal vesicle (GV) – where a circular vesicle (oocyte nucleus) is visible inside the oocyte cytoplasm (in prophase I of meiosis); germinal vesicle breakdown or first meiosis (GVBD/MI) – stage where the vesicle is no more discernible; second meiosis (MII) – the vesicle is not observable and the first polar body is already present.

Figure 5: Schematic illustration of the major steps of the oocyte maturation (modified from Swain and Pool, 2008)

Immunohistochemistry:

In order to evaluate the proportion of granulosa cells in proliferation, an immunohistochemistry protocol was optimised for the Ki-67 staining, as it is an extensively used cell proliferation marker. After deparaffinisation and rehydration, slides were rinsed in PBS and then transferred into a citrate buffer, placed in the

microwave for 5 min, cooled down for 5 min and rinsed again in PBS. An endogenous peroxidase inhibition has followed, using 3% H2O2/methanol solution for 30 min and then washing in tap and distilled water. Normal goat serum (5% NGS) in PBS was added for 1 hour at room temperature. After rinsing with PBS, all endogenous biotin, biotin receptors, and avidin binding sites of the sections were blocked, by covering the sections with avidin/biotin blocking kit drops, for 15 min (SP-2001, Vector Laboratories, US) and again washed in PBS. The sections were then incubated at 4ºC overnight with rabbit monoclonal antibody against Ki-67 (VP-RM04, Vector Laboratories, US), diluted 1:200/1:400 in 5% NGS in PBS. Following the primary antibody incubation, the slides were washed in PBS and the secondary antibody (Biotinylated Goat Anti-Rabbit IgG, dilution 1:300, BA-1000, Vector Laboratories, US) was added for a period of 1 hour at room temperature. After PBS rinsing, an Avidin/Biotinylated Enzyme Complex (PK-6100, VECTASTAIN *Elite* ABC kit, Vector Laboratories, USA) was used for an incubation time of 30 min, followed by a peroxidase activity development produced by a 3,3'-diaminobenzidine solution (SK-4100, DAB Peroxidase Substrate Kit, Vector Laboratories, USA) for 5 min. The slides were finally washed in tap and distilled water, counter-stained with toluidine blue, dehydrated and mounted. The negative control was processed identically, with the primary antibody being replaced by an IgG rabbit (dilution 1:5000).

Hormonal analyses

Serum levels of progesterone were determined using an automatic electro-chemiluminiscent technique (Model E170, Roche, Mannheim, Germany), diluted 1:3 in Progesterone standard diluent (Diagnostic Products Corporation, USA). The sensitivity and inter-assay coefficient of variations were, respectively, 0.25 ng/ml and <5%.

Statistics

Statistical analyses were performed using SPSS or Vassar Stats website. The follicular count was compared using ANOVA tests. Follicular maturation was compared using Fisher's exact test. Values of $p<0.05$ indicated statistical significance.

Experimental design

The experimental design was partially reproduced and adapted from a previous study (Meirow, 2004). The two first experiments were performed to evaluate the two chosen GnRHa and Cy doses, previously described in the literature.

Three experiments were performed to evaluate the histological aspect and the follicular count, to execute immunohistological tests and follicular *in vitro* cultures (in total: six mice/condition). A fourth assay was made only for follicular culture, using both ovaries of all mice, in order to achieve a greater number of follicles per condition (external controls were used this time, to histologically testify that the treatments were well performed) (two mice/condition);

Three additional assays were performed to evaluate the long-term fertility effect (in total: six mice/condition), with individual mating of the treated females with a F1 hybrid male. As this study was designed to evaluate the efficiency of GnRHa to prevent future fertility decline induced by chemotherapy, the GnRHa-control groups were not included.

Results

None of the mice died during the treatments, and only a female from the follow-up assay died after the birth of its 3rd litter.

Preliminary tests

The two first series were performed to evaluate if the GnRHa and Cy concentrations used had a similar effect as previously described when compared with the controls. Four treatment conditions were included in each treatment group (ANT and AGO). In each of the assays, two female mice were used per condition, and both of its ovaries were counted separately (total n=16; n/condition=2; n ovaries/condition=4). The follicular count was regrouped into two classes: initial stage (primordial + primary) and growing stage (secondary + early antral + antral) (Figure 6).

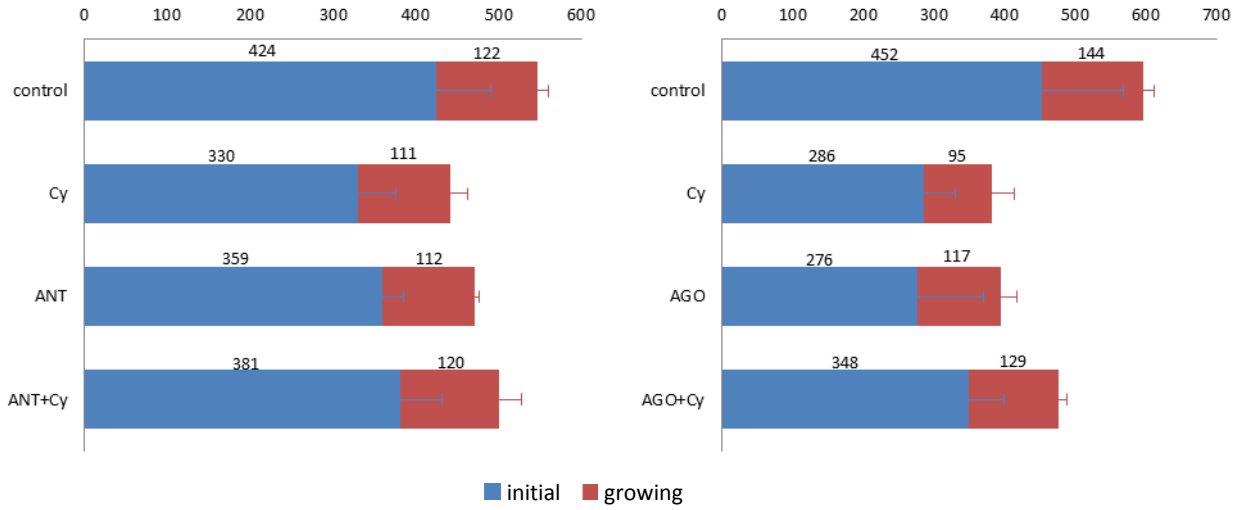


Figure 6: Total number of follicle count according to the treatment

In the ANT assay, the mean (±SD) number of follicles of the control group in the initial stage was 424 ± 65 and in the growing stage 122 ± 14. When compared with the control, the chemotherapy-control group presented a decrease of 22% in the initial stage and 9% in the growing stage ones. Concerning the ANT-control group and the co-treated ANT+Cy group, they showed, respectively, a decrease of 15% and 10% of the initial stage (p=0.177), and 8% and 2% of the growing stage, respectively (p=0.81) (Figure 7). Nevertheless, when comparing the groups treated with Cy alone vs ANT+Cy, an increase of 15% and 8% of the follicular population at initial and growing stage, respectively, was observed.

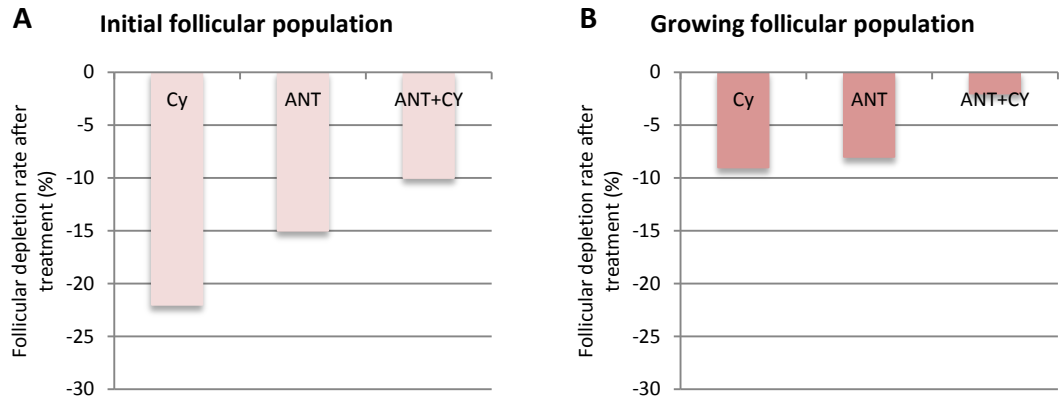


Figure 7: Depletion rate of the initial stage (A) and growing stage (B) ovarian follicular population after the first preliminary ANT treatment assay, comparing with the control.

In the AGO assay, the control group revealed in the initial stage 452 ± 117 follicles, in the final stage 144 ± 16. When compared with the control, the chemotherapy-control group presented a decrease of 37% in the initial stage and 34% in the growing stage. Concerning the AGO-control and the co-treated AGO+Cy groups, they showed, respectively, a decrease of 39% and 23% of the initial population (p=0.078) and of 19% and 11% of the growing population (p=0.1) respectively, when compared with the control group (Figure 8). When the groups treated with Cy alone vs AGO+Cy were compared, an increase of 23% and 35% of the follicular population at initial and growing stage respectively was observed.

Despite the absence of significance due to the limited number of ovaries and the inter-variability, we considered the Cy doses as sufficiently efficient to continue the experiment.

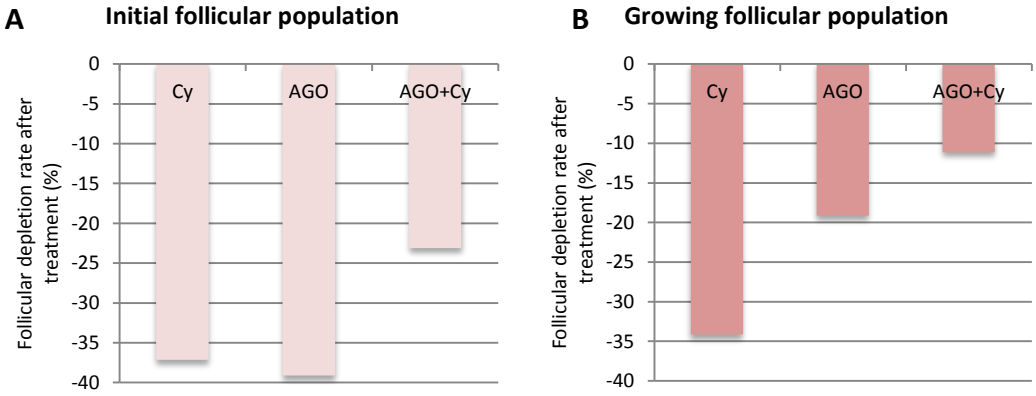


Figure 8: Depletion rate of the initial stage (A) and growing stage (B) ovarian follicular population after the first preliminary AGO treatment assay, comparing with the control.

Comparison of GnRH agonist and antagonist effect on the follicular reserve

These results encompass the analysis of three series, each one including two mice per condition with the follicular count of one ovary/mouse, the other ovary was used for follicular culture (n total=36; n/condition=6).

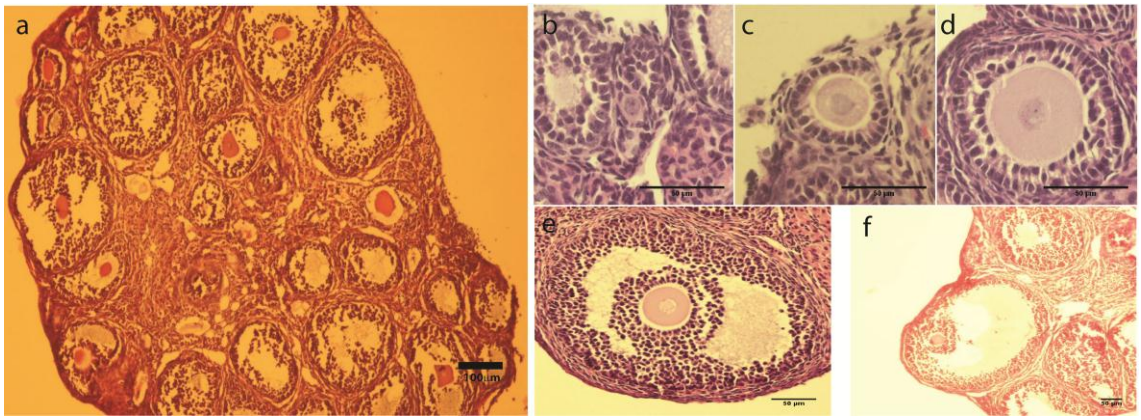


Figure 9: Histological mouse ovary sections with haematoxylin and eosin staining. Entire ovary (a); primordial follicle (b); primary follicle (c); secondary follicle (d); early antral follicle (e); antral follicle (f). Scale bar: 50 µm (section a – scale bar: 100 µm).

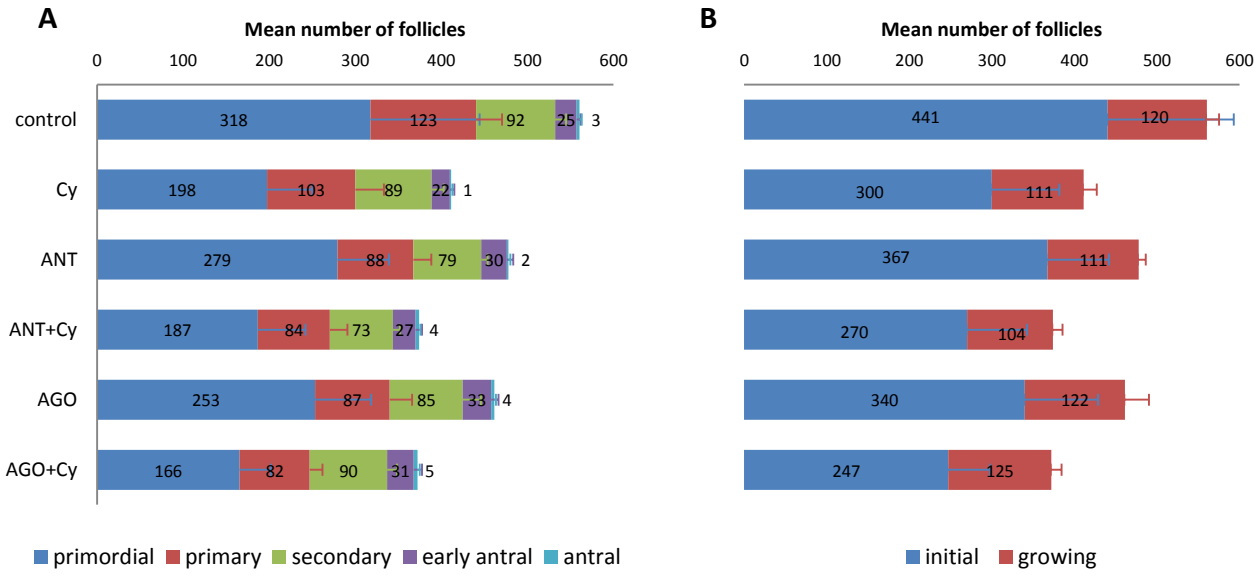


Figure 10: Mean follicular count for each stage according to the treatment conditions. Five-stage classification (A) and re-grouped two-stage classification (B).

The control group had the highest mean of follicles at the initial stages: 441 ± 153; but no significant difference was observed with the mice treated with GnRHa alone: ANT – 367 ± 74; AGO – 340 ± 89 (p=0.358) (see Figure 10). The follicular depletion rate compared to control was 17% and 8% for the ANT group, and of 23% and 1% in the AGO group for the initial and growing population respectively (Figure 11). Regarding the Cy-treated mice, an important reduction in the follicular reserve is exhibited in all three groups, when compared with the control group: Cy – 300 ± 82 (-32%), ANT+Cy – 270 ± 73 (-39%), AGO+Cy – 247 ± 51 (-44%), reaching statistical significance for the last one (p=0.022). No significant difference was observed between conditions regarding the counting of follicles in the growing stage population (Figure 11B).

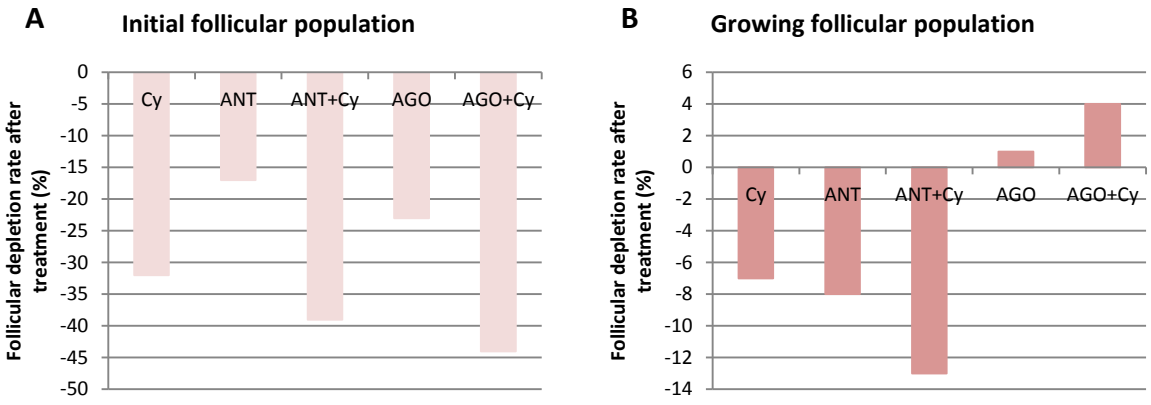


Figure 11: Depletion rate of the initial stage (A) and growing stage (B) ovarian follicular population after GnRHa treatments, comparing with the control.

The proportion of each follicular stage is conserved through all treatment conditions. The relative percentages range between 66-78% for the initial stage follicles and 22-34% for the growing stage ones (Figure 12).

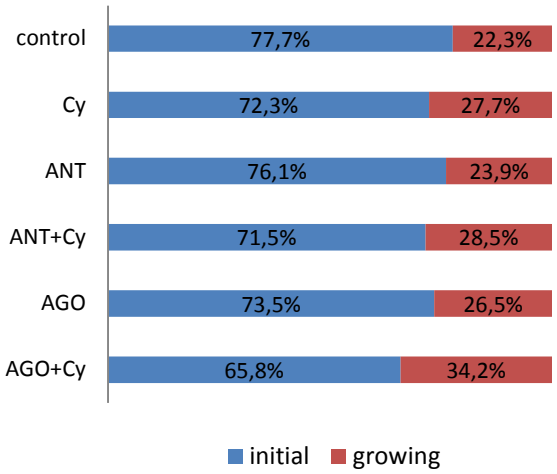


Figure 12: Mean proportion of follicles for each stage according to the treatment conditions.

Immunohistochemistry

Preliminary tests showed that the Ki-67 staining seem to preferentially mark the granulosa cells in proliferation, from growing follicles, such as secondary (Figure 13) and early antral follicles. Ongoing experiments to evaluate a possible difference between treatment groups are being performed.

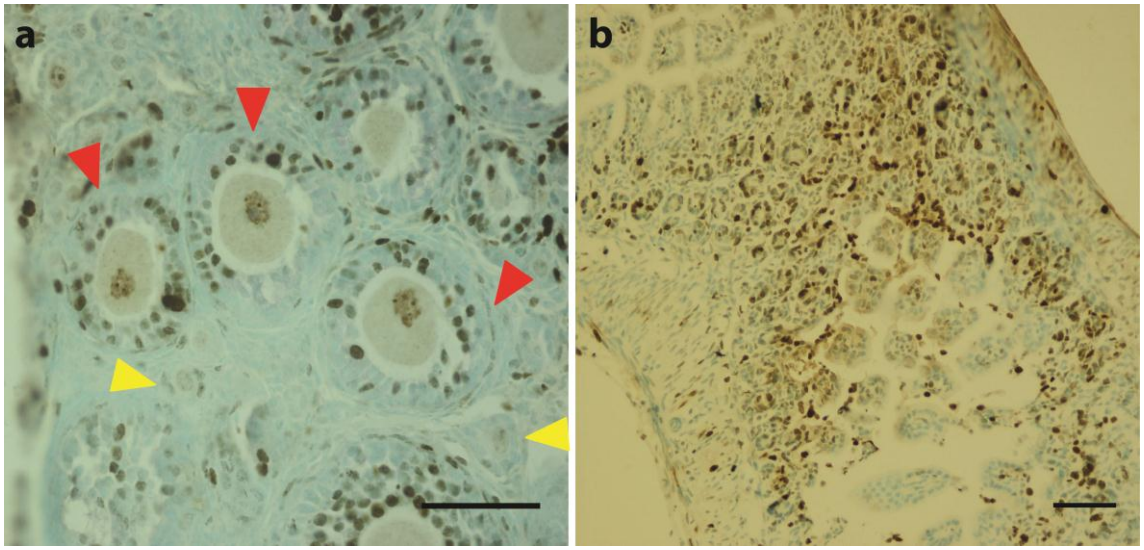


Figure 13: Ki-67 immunostaining on a control mouse ovary. Red arrows indicate secondary follicles with stained granulosa cells. Yellow arrows indicate non-stained primary follicles (a). Mouse intestine with epithelial cells stained (b). Scale bars: 100 µm.

Follicular in vitro culture

Following the mice sacrifice and ovariectomy, one ovary from each mouse was carefully dissected (exception made in the fourth assay, where both ovaries from each mouse were used), in order to collect the preantral follicles, which were cultured separately in individual drops of medium. A total of 585 follicles from the different treatment conditions were cultured (56 ovaries from 48 mice; i.e., 8 per condition). In vitro follicular growth is illustrated in the Figure 14.

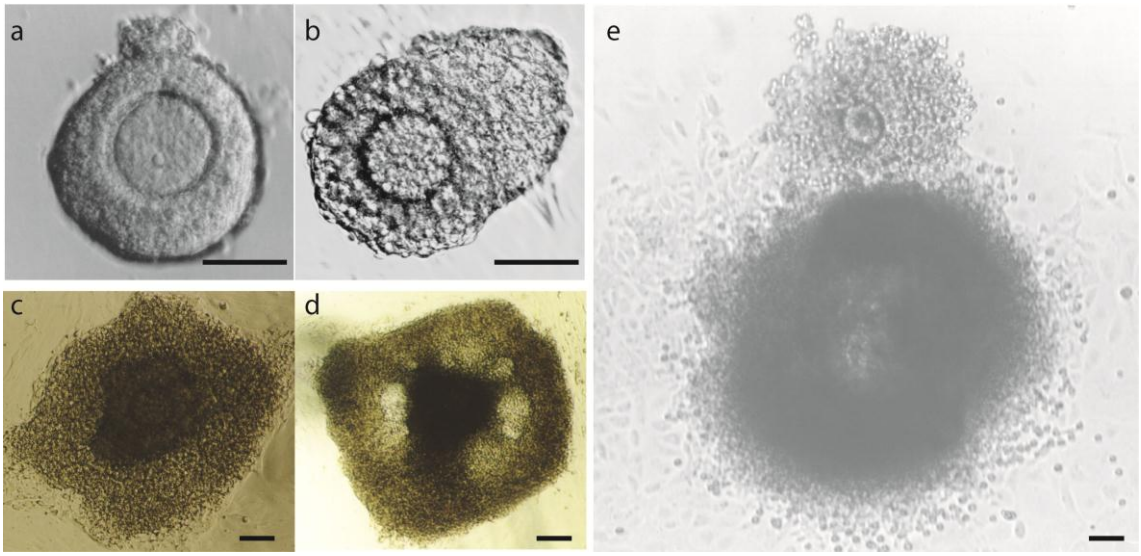
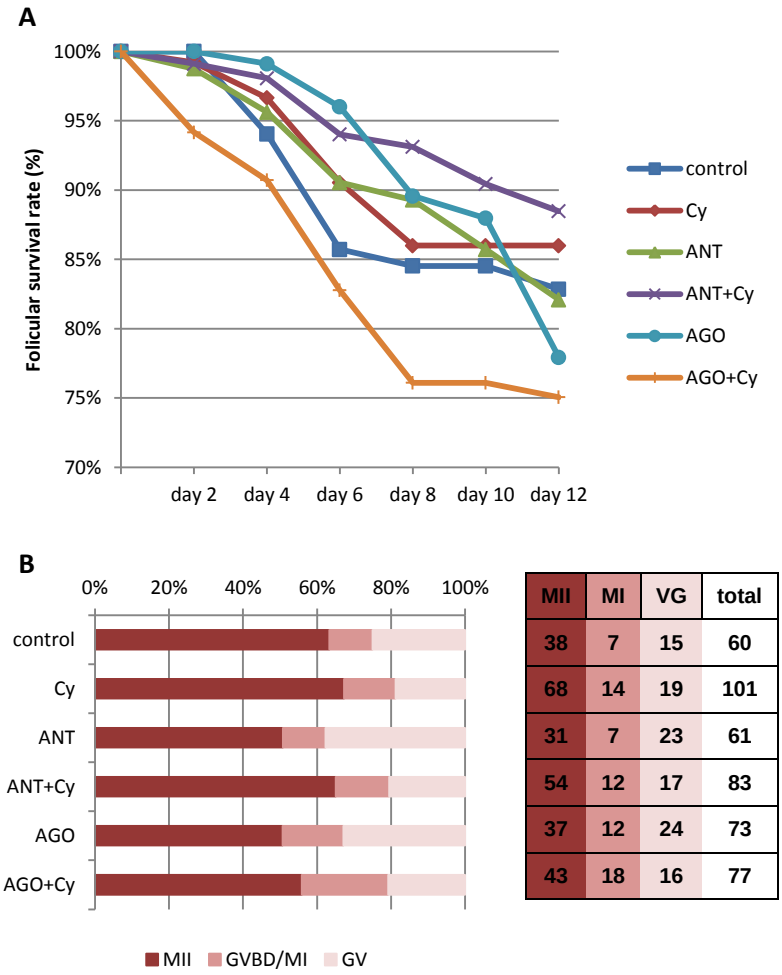


Figure 14: Follicular *in vitro* maturation. Follicles in culture day 2 (a), day 4 (b), day 10 (c), day 12 (d) and day 13 (e). This last presents the oocyte already outside the granulosa cell layers, after finished the maturation process, but still surrounded by cumulus cells. Scale bar: 50 µm.

The follicles were observed every two days to report the survival rate. A degenerated follicle was characterised, preferably by a complete oocyte expulsion followed by the detachment or demise of the granulosa cell layers, by a permanent wrinkled surface, or by the non-attachment to the bottom of the culture dish after a few days. No difference was observed between the follicular survival rates in the different treatment conditions (see Figure 15A).



MII	MI	VG	total
38	7	15	60
68	14	19	101
31	7	23	61
54	12	17	83
37	12	24	73
43	18	16	77

Figure 15: Follicles survival rate when cultured *in vitro*, depending on the treatment (A). Proportion of oocyte nuclear stages on Meiosis II (MII), Meiosis I (MI) or germinal vesicle (GV), in the different conditions (B).

No significant difference was observed in the oocytes maturation rate at day 13. Once the control group presented 63% of oocytes in the MII stage (see Figure 15B) and all the other ones ranging between 51-67%, one can conclude that the previous treatment condition have no impact on the capacity of the survival follicles to mature *in vitro*.

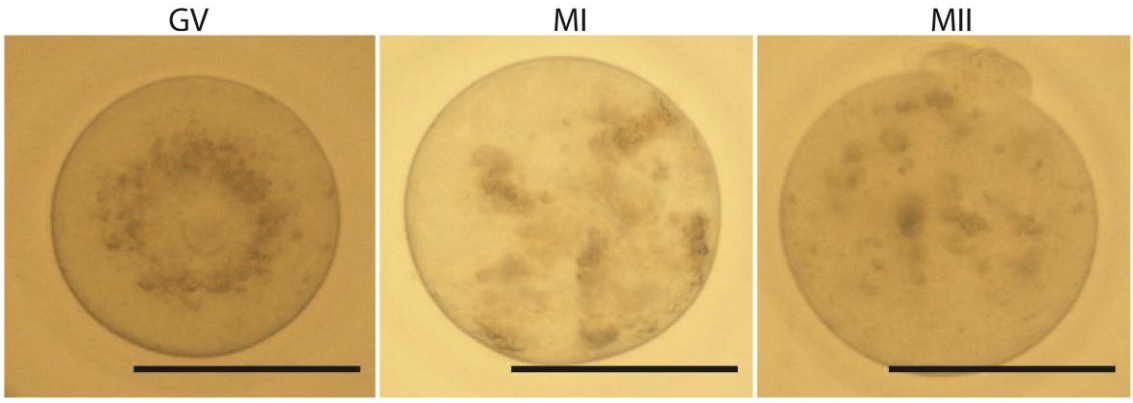


Figure 16: Isolated oocytes at three maturation stages: germinal vesicle (GV), Meiosis I (MI) and Meiosis II (MII). Scale bar: 50 µm.

Hormonal analysis

With the collected serum from the different condition culture drops of day 13, the levels of progesterone secreted to medium were measured. The control group had a 24 hour-average production of 2,89 ng/mL of progesterone (Figure 17). The former groups did not show statistically significant differences, although the GnRHa-alone treatments appear with an interesting smaller progesterone production in response to hCG.

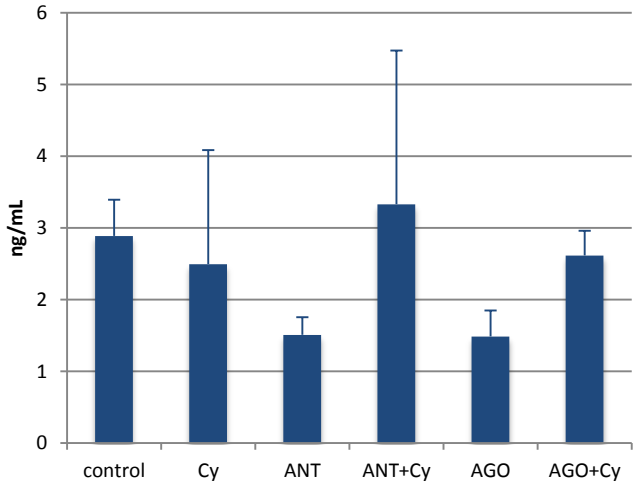


Figure 17: Progesterone 24 hour-average production in control and treated conditions (from day 12 to day 13).

Fertility follow-up

Three injection series were performed to evaluate the long-term mouse pups rate after treatments. Hence, after the treatments have ended, all the females were individually mated. This analysis included the mean litter size of 36 mice couples (6 couples per condition). As the series were not done all at once, the results from the fourth litter (including) and further on, presented in Figure 18 belong only to two couples (the first two treated females for each condition).

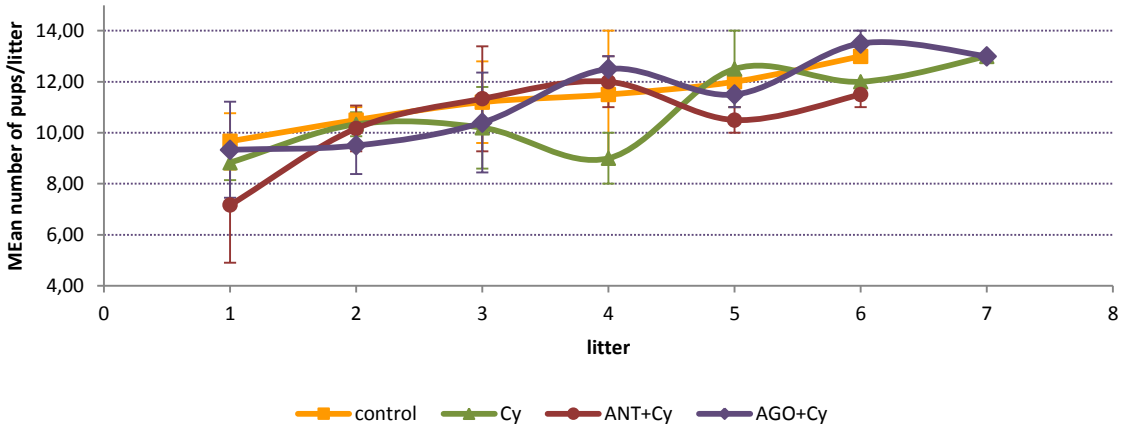


Figure 18: Variation of the average number of mouse pups per litter in several analysed conditions.

The average number of new-borns per litter is very close between all conditions, and is also increasing (until the conclusion of this work). No significant differences are noted, therefore suggesting that the treatments have no effect on the mice fertility, at least in short/medium-term. Moreover, the average number of days between births was fairly close, with an overall mean of new mouse pups born every 23 days.

Discussion

Although the accomplishment of new cancer treatments could significantly improve the lifespan of patients, the corresponding improvement in the quality of life cannot always be achieved. Specifically, major concerns are addressed regarding the female population, which rightfully intends the safeguarding of their fertility.

Numerous studies have confirmed the irreparable ovarian toxicity caused by alkylating chemotherapy regimens (Mattison *e al.,* 1981; Meirow, 1999; Oktem *et al.,* 2007). These treatments have, almost constantly, severe side effects, leading to the development of premature ovarian failure (POF) during reproductive-age, and consequently to the impossibility to conceive (Blumenfeld *et al.,* 2008; Sonmezer *et al.,* 2004). Therefore, different options have been proposed to preserve the fertility of these young patients, including pharmacological protection.

The present study aims to evaluate the validity of the mice model to further compare the mechanisms of action and the efficiency of the GnRh analogues to prevent chemotherapy-induced ovarian damage. It must be highlighted that this study and this report are the beginning of an ongoing project, and therefore the small amount of individuals, must be taken into consideration in the interpretation of the results as well as in the validity of the statistical analysis.

In the present study, we confirmed the gonadotoxic effect of Cyclophosphamide. The number of primordial and primary follicles was reduced when Cy is administered compared to the control, whatever the mice were co-treated with GnRHa or not. While the drug- and dose-dependent manner whereby cancer treatments affect the ovarian reserve is generally accepted (Meirow, 2001a, 2001b; Arnon *et al.,* 2001), it must be emphasized that when we pooled our experiments, a significant depletion of the follicles was confirmed (-30,7%, p=0.002), but still did not reach half of the ovarian reserve as predicted for the same Cy concentration using in other studies (Meirow, 1999; Meirow *et al.,* 2004). The difference may be related to the follicular count methodology but also to technical issues, as for example, the Cy dilution and ip injection. Known to be an extremely perishable and delicate compound (photosensitive), Cy may also crystalize in the peritoneum at the time of the injection, so it does not disseminate properly into the bloodstream, resulting in a weak effect. Nevertheless, these preliminary results seem to support the hypothesis that alkylating agents harm the growing follicles, inducing a subsequent recruitment of the initial follicular pool into the growing process (Blumenfeld *et al.,* 2008). This mechanism of action has been reported as the “burn out” action of chemotherapeutical agents.

In order to avoid the damage created by chemotherapy, some authors have proposed a non-invasive procedure able to suppress the hypothalamic-pituitary-ovarian axis, thus interrupting the follicular growing process and consequently protecting the ovarian reserve, and so the fertility. This potential strategy is the concomitant administration of GnRHa along with chemotherapy. The effect of GnRHa in parallel to chemotherapy has been examined both in humans and animal models (Ataya *et al.,* 1985; Ataya *et al.,* 1995; Blumenfeld *et al.,* 2008; Del Mastro *et al.,* 2011; Meirow *et al.,* 2004; Tan *et al.,* 2010; Whitehead *et al.,* 2011). In humans, Blumenfeld *et al.,* have reported lower rates of POF in women co-treated with GnRHa (11.1%) compared to the chemotherapy-only group (55%) in an observational study (Blumenfeld *et al.,* 2008). Among GnRHa co-treated women, 22% get pregnant after chemotherapy, compared with 14% of those without GnRH agonist therapy (Clowse *et al.,* 2009). Most of these studies were observational or/and included important bias maintaining the debate around the real efficiency of this treatment and its mechanism of action. Despite some promising effects of GnRH analogues, there are indeed many variables that are not uniform between trials, thus becoming difficult to evaluate, to compare them and therefore to make trustful conclusions: randomised/non-randomised clinical trials, sample size, follow-up time, GnRHa treatment, chemotherapy regimen, less sensitive markers (pregnancy rate, resumption of menstruation, levels of serum sex steroids and gonadotropins), etc. Few recent randomized studies still show contradictory results. Among them, some did not show difference in the premature ovarian failure rate after chemotherapy with or without GnRha co-treatment (Behringer *et al.,* 2010; Demeestere *et al.*, 2012), resulting in the persistence of the controversies after more than 20 years of investigations.

We are aware of the incomplete understanding of all the effects and mechanisms underlying this kind of co-treatments, or even the existence of a true positive effect. While the profound inhibition of gonadotropins secretion was reported as the main mechanism of protection, no clear evidence supporting this hypothesis is available. Moreover, once there is no expression of FSH receptors on primordial follicles, this mechanism cannot be explained by its direct effect on the ovarian follicles (Oktay *et al.,* 1997). Another proposed mechanism by which GnRHa may provide ovarian protection is through a decrease in ovarian blood flow, consequently causing a reduction in the amount of chemotherapy reaching the ovary (Reinsch *et al.,* 1994). However, studies on the effect of GnRHa on blood flow are still few and contradictory (Kitajima *et al.,* 2006).

Hence, it is still much more critical to consider that there are still many questions to be addressed and answered, so more animal model studies are urgently

needed. However, several studies have already contributed to increase controversy around the GnRHa putative ovarian protection during chemotherapy. In 1995, a non-human primate experiment demonstrated that GnRHa significantly decrease the follicular depletion associated with gonadotoxic cyclophosphamide treatment (Ataya *et al.,* 1995). Bokser *et al.,* using female rats suggested that treatment with GnRHa microcapsules before and during chemotherapy prevented the ovarian injury caused by Cy (Bokser *et al.,* 1990). Meirow and his colleagues demonstrated the same conclusions in the mouse, using the GnRH antagonist cetrorelix (Meirow *et al.,* 2004). On the other hand, numerous other authors, using rat and mouse model, have advocated that this protection was not present or not statistically significant (Danforth *et al.,* 2005; Gosden *et al.,* 1997; Montz *et al.,* 1991).

The number of follicles, especially primordial follicles, is the more accurate estimation of the ovarian reserve (Oktem *et al.,* 2007). Thus, presently, quantitative measurement of ovarian follicles in different stages appears as the best way to validate or not the protective effect of a GnRH analogue, besides some incongruities and difficulties sometimes associated with the counting (Tilly, 2003).

Our results seem to indicate a non-preventive effect of GnRHa (both agonist and antagonist) along a follicle disruption produced by a Cy treatment. Furthermore, the mice treated with GnRHa alone showed also a decrease on the initial follicles pool: -17% and -23% in the GnRH antagonist and in the agonist groups respectively, when compared with the control. Despite they did not reach significance, these results may suggest a possible negative effect of the treatment on the ovary. Furthermore, the proportion of growing follicles was similar whatever the treatment. Surprisingly, these results suggest that GnRHa treatment might not totally inhibit the recruitment of the growing follicular pool. The presence of growing follicles was not mentioned in the previous published studies using similar protocol and other hormonal tests confirming that ovarian suppression was until now poorly investigated. The ovarian function of the treated mice have thus to be further evaluated using analysis of ovarian reserve markers, such as serum levels of follicle-stimulating hormone (FSH), estradiol, etc. (Luchtman Singh *et al.,* 2007) in the blood samples previously collected. In addition, histological analysis will further indicate the proliferation capacity and the viability of these follicles. Therefore, we planned to evaluate the proliferation and the apoptosis associated to the granulosa cells, respectively testing the activity/presence of the proteins Ki-67 and caspase-3.

However, we already tested the capacity of these secondary follicles, isolated after treatment, to develop *in vitro* until pre-ovulatory stage. This individual 2-D culture system was used in our laboratory as a model to follow the development potential of

each secondary follicle and to test subsequent oocyte maturation competence (Demeestere *et al.,* 2002; Demeestere *et al.*, 2004). In order to compare each treatment effect, we tried to isolate a similar number of follicles; however, a non-significant difference in the survival rate was observed in the different groups. Furthermore, oocytes grown *in vitro* acquired similar maturation competence and follicles are able to secrete progesterone in response to hCG/EGF. Besides the non-significant differences (maybe due to the small size of the sample), it seems however that both GnRHa alone reduced subsequent progesterone production in response to hCG. Altogether, these results suggested that the folliculogenesis was not altered anymore by the chemotherapy treatment after one week. On the other hand, they also showed that analogues treatment did not affect the development potential of the follicles, at least until secondary stage. These results confirmed previous studies on FSHβ knockout model showing that the recruitment of the initial follicular population was not completely inhibited in the absence of FSH (Demeestere *et al.,* 2002). However, the follicular growth is delayed and in adults, *Fshβ-/-* individuals contained fewer secondary follicles than wild-type individuals and no large antral follicles. These observations may be more consistent with what is observed in human during GnRH analogues treatment. Further investigations are thus necessary to demonstrate the efficiency of the treatment and the validity of the mice model for such studies.

As mentioned above, another question addressed in our study was the fertility of the treated females. If the Cy-treated females show a decrease in the ovarian reserve at histological level, probably due to the constant recruitment and demise of the growing follicles, one can predict that the reproductive capacity of these females would be severely affected. So, we hypothesized that the number of mice per litter would start to decrease quicker in these females than in the control. However, our treated-mice did not show any decline of their fertility even after seven litters. As such, the experiment should continue, in order to verify if the expected alteration of the fertility will arise.

In conclusion, despite this study is still ongoing, the results obtained addressed many question regarding this model and further emphasize the necessity to validate it before drawing conclusions and extrapolating them in human. At this point, our animal study did not show any protective effect of either GnRH agonist or antagonist on chemotherapy-induced damage in the ovaries. However, the efficiency of this treatment protocol to induce profound inhibition of gonadotropins secretion as observed in human, and therefore, of the follicular growth process must be further confirmed. Moreover, the doses of the chemotherapy should be adapted in order to increase the ovarian damage without inducing immediate irreversible sterility.

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